FEBS Letters 589 (2015) 1966–1974

FEBS Letters

journal homepage: www.FEBSLetters.org

The DRBD13 RNA binding protein is involved in the insect-stage differentiation process of *Trypanosoma brucei*

Bhaskar Anand Jha [1], Vahid H. Gazestani [1], Chun Wai Yip, Reza Salavati *

Institute of Parasitology, McGill University, 21,111 Lakeshore Road, Ste. Anne de Bellevue, Quebec H9X3V9, Canada

ARTICLE INFO

Article history:
Received 15 February 2015
Revised 21 April 2015
Accepted 20 May 2015
Available online 28 May 2015

Edited by Michael Ibba

Keywords:
DRBD13
Post transcriptional gene regulatory program
Insect stage differentiation process
Trypanosoma brucei

ABSTRACT

DRBD13 RNA-binding protein (RBP) regulates the abundance of AU-rich element (ARE)-containing transcripts in trypanosomes. Here we show that DRBD13 regulates RBP6, the developmentally critical protein in trypanosomatids. We also show DRBD13-specific regulation of transcripts encoding cell surface coat proteins including GPEET2, variable surface glycoprotein (VSG) and invariant surface glycoprotein (ISG). Accordingly, alteration in DRBD13 levels leads to changes in the target mRNA abundance and parasite morphology. The high consistency of the observed phenotype with known cell membrane exchanges that occur during progression of *T. brucei* through the insect stage of its life cycle suggests that DRBD13 is an important regulator in this largely unknown developmental process.

1. Introduction

Trypanosoma brucei is a unicellular protozoan parasite and the causative agent of sleeping sickness in humans and nagana in cattle [1]. The parasite is transmitted between different mammalian hosts by an insect vector "tse-tse fly" [2]. To complete its life cycle in the two hosts, trypanosomes go through several morphological and molecular changes in response to the changing conditions [2,3]. In the insect vector for instance, trypanosomes encounter and adapt to different environments in the fly midgut, proventriculus and finally in the salivary glands [4]. Two of the predominant developmental forms cultured in laboratory are blood-stream form (BS; residing in the mammalian host) and procyclic form (PC; in the insect vector). While the cell surface of BS is covered with VSG [5] and less abundant ISGs [6,7], PC trypanosomes are coated with family of glycoproteins called procyclins [8]. During differentiation from PC to BS, trypanosomes reacquire the VSG coat and lose their procyclins in the fly salivary gland at a stage termed metacyclic (MC; the only infective form in trypanosome life cycle) [3,4]. Although trypanosomes are very successful in adapting to changing conditions in different hosts, the parasites have almost no transcriptional control of gene expression and heavily rely on the post-transcriptional gene regulation [9]. Interaction between the *cis* regulatory elements in the 3′ untranslated region (UTR) of mRNAs and the *trans* acting RBPs are indispensable for this kind of regulation [10,11]. In higher eukaryotes, several ARE binding proteins (AUBPs), with multiple regulatory roles on the ARE containing mRNAs, have been discovered. While some of them stabilize ARE containing transcripts [12,13], others can mediate degradation [14–17] or translational repression of their ARE-containing target RNAs [18,19]. In case of trypanosomes, our lab previously reported the presence of AREs in the 3′UTR of developmentally regulated mRNAs and found three putative AUBPs that regulate the fate of these ARE-containing transcripts [20]. Here, we have characterized one of these three AUBPs, called DRBD13, which contains two RNA recognition motifs (RRMs) [21]. We show that the tagged DRBD13 is localized in the cytoplasm of PC trypanosomes. DRBD13 level is tightly regulated in the PC stage, as DRBD13-myc over-expression as well as *DRBD13 RNAi* is deleterious to the parasite's growth. Analysis of data obtained from three high throughput experiments on this protein, suggested the regulatory role of DRBD13 protein on transcripts that encode cell membrane proteins. Consistently, both *DRBD13 RNAi* and an ectopic expression of tagged DRBD13 led to changes in the expression level of membrane associated proteins and, accordingly, severe changes in the parasite's cell morphology. Additionally, the RIP-seq data suggested binding of the *RBP6* transcript to DRBD13

Author contributions: BAJ, VHG, CWP, and RS designed research; BAJ, VHG, and CWP performed research; BAJ, VHG, CWP, and RS analyzed data; BAJ, VHG, and RS wrote the paper.

* Corresponding author. Fax: +1 514 398 7857.
E-mail address: reza.salavati@mcgill.ca (R. Salavati).
[1] These authors have equal contribution to this study.

http://dx.doi.org/10.1016/j.febslet.2015.05.036

protein. Involvement of RBP6 in differentiation from the PC to MC stage of life cycle has already been demonstrated [22]. Here we show that *DRBD13 RNAi* leads to an up-regulation of *RBP6* mRNA. These results highlight the importance of DRBD13 as a necessary factor for normal growth of the insect form of trypanosomes and the protein's potential involvement in the developmental process of the parasite in the fly.

2. Materials and methods

2.1. Trypanosome culture

Culturing and transfection of trypanosomes (Lister 427 strain expressing the tetracycline (tet) repressor from pHD1313 and 29–13) were performed as described in [20].

2.2. Plasmid constructs

Oligonucleotides and plasmids prepared for this study are listed in Tables 1 and 2, respectively. For ectopic over-expression in 1313-PC cells, DRBD13 ORF was cloned in pHD1700 (C-terminal myc tagging; *Hind*III and *Hpa*I) and pHD1701 (N-terminal myc tagging; *Apa*I and *Bam*HI), followed by linearization by *NotI* and transfection [23]. For tethering experiments *DRBD13* ORF was cloned in pHD1743 (*Apa*I and *Bam*HI) [24], so that the resulting plasmid encoded a fusion protein, λN-DRBD13-myc. The linearized plasmid was transfected into two different cell lines constitutively expressing CAT (*chloramphenicol acetyl transferase*) reporter lacking, and harboring, five copies of the λN-peptide recognition site "boxB" – in its 3′UTR (pHD1991 and pHD2277, respectively) [24].

2.3. Indirect immunofluorescent staining and Western blotting

For immunofluorescent assay, the steps of slide preparation were performed as previously described [25], and the images were taken using the Nikon eclipse E800 microscope. For Western blotting, we used: anti-myc (Life technologies, #A21280), anti-CAT (Sigma, #C9336) and antibodies against the small ribosomal subunit protein S9 and phosphoglycerate kinase (PGK).

2.4. Northern blotting

Parasites were harvested and RNA was prepared using Trizol reagent (Ambion, CA, USA). Total RNA (10 μg) was run on a denaturing formaldehyde gel, followed by blotting. DNA probes (see Table 1 for PCR primers) were prepared using the Prime-IT Random Primer Labeling Kit (Stratagene) and hybridized at 65 °C.

2.5. Semi-quantitative reverse transcription PCR

RNA was treated with DNase using Turbo DNA-free Kit (Life Technologies, AM1907). cDNAs were prepared from 4.5 μg RNA using random hexamers and Taqman Reverse transcription reagents (Life Technologies, N8080234) in a 30 μL reaction; subsequently diluted 5-fold in water (120 μL H_2O). Control reactions without reverse transcriptase (RT) were used to confirm elimination of genomic DNA contamination. Semi-quantitative PCR was performed in a 20 μL reaction containing 50 ng of cDNA, 1× GoTaq Green Master Mix (Promega, M712), and 1 μM of each forward and reverse primers, (primers described in [22]). The PCR reactions were visualized on a 1% agarose gel.

2.6. Cell fractionation and polysome purification

Tetracycline was added to approximately 100 mL of 1313-DRBD13-myc PC trypanosomes growing at a density of 5×10^6 cells/mL. After 3 h of DRBD13-myc induction, the cells were fractionated as described in [26] followed by polysome preparation as in [27]. Western blots were incubated in antibodies against myc tag and small ribosomal subunit protein S9.

Table 1
Primers used in this study.

Primer name	Primer seq	Restriction site
DRBD13_Nmyc_Fw	agctgggcccTGACAGACCACCAAGTCATCTGC	*Apa*I
DRBD13_Nmyc_Rev	agctggatccTCAACCTACACGCATGGTGGGA	*Bam*HI
DRBD13_Cmyc_Fw	agctaagcttATGACAGACCACCAAGTCATCT	*Hind*III
DRBD13_Cmyc_Rev	agctgttaacACCTACACGCATGGTGGGAGTC	*Hpa*I
DRBD13_tethering_Fw	agctgggcccATGACAGACCACCAAGTCATCT	*Apa*I
DRBD13_tethering_Rev	agctggatccACCTACACGCATGGTGGGAGTC	*Bam*HI
Splice leader Fw	GACTAGTTTCTGTACTAT [22]	
VSG 3′UTR reverse	CCGGGTACCGTGTTAAAATATATC [22]	
ISG Fw	CAGGTGGAAGTTGGTATTGA	
ISG Rev	TCCTTATTTACACTCGCTGT	
GPI-PLC Fw	CTCTGCTGCGTTTGTGACTG	
GPI-PLC Rev	TTTCAAACACCGTCCCCCTC	
DRBD13_Probe_Fw	GCAACCTCTTCATATCGGGA	
DRBD13_Probe_Rev	CGCCTGCTTACCAGAAGAAC	
RBP6_Probe_Fw	CAACAGCCGTATCATCCCTT	
RBP6_Probe_Rev	ACTCATTCGCTCCACAGCTT	
CAT_Probe_Fw	ATCCCAATGGCATCGTAAAG	
CAT_Probe_Rev	ATCACAGACGGCATGATGAA	

Fw: forward primer; Rev: reverse primer.
Restriction sites are underlined; complementary sequences of primers are in uppercase.

Table 2
Plasmids used in this study.

Plasmid name	Description	Restriction sites
C-myc DRBD13	Ectopic over-expression of DRBD13-myc; pHD1700 + DRBD13 [23]	*Hind*III/*Hpa*I
N-myc DRBD13	Ectopic over-expression of myc-DRBD13; pHD1701 + DRBD13 [23]	*Apa*I/*Bam*HI
DRBD13 tethering	Ectopic expression on λN-DRBD13-myc; pHD1743 + DRBD13 [24]	*Apa*I/*Bam*HI
pHD1991	Reporter vector without boxB (recognition sequence for λ peptide) in the 3′UTR downstream of CAT ORF [24]	
pHD2277	Reporter vector with five boxB (recognition sequence for λ peptide) in the 3′UTR downstream of CAT ORF [24]	

3. Results and discussion

3.1. Identifying the direct targets of DRBD13

The DRBD13 protein (*Tb*927.8.6650) is 666 amino acids (aa) long, and has an RNA binding region containing two closely located RRM domains (Fig. 1A). Interestingly, the 5′ and 3′ flanking sequences of the RNA binding region (270aa and 200aa long, respectively) are unique to *T. brucei* subspecies and cannot be found in other trypanosomatid organisms, suggesting its unique function/interacting partners in *T. brucei*. Previous reports have demonstrated the functionality of DRBD13 protein in the PC life stage of the organism [20] and its upregulation in the stationary phase (stationary phase presumably resembles the metacyclic life stage of the parasite) [28]. As a first step towards determining the DRBD13 mRNA targets, we re-analyzed the data obtained from three high-throughput experiments on the RBP (Fig. 1B): (1) transcriptome changes in response to DRBD13 over-expression; (2) transcriptome changes in response to *DRBD13* depletion; and (3) high-throughput sequencing of transcripts co-purified with DRBD13 tandem affinity purification (RIP-seq) [20]. Due to the complementary nature of these approaches, their integration leads to the removal of false positive results (*e.g.*, secondary/indirect effects) and gaining of a thorough picture on the biological function and direct targets of the protein under investigation. Analysis of the transcriptome data suggested that DRBD13 acts as a negative regulator of transcripts encoding ribosomal and cell membrane proteins and the proteins involved in transport (Fig. 1C). Additionally, the analysis of RIP-seq data indicated that DRBD13 preferentially associates with the transcripts encoding cell membrane associated proteins and also proteins involved in the post-translational modifications (Fig. 1C). RIP-seq data also demonstrated that *RBP6* (*Tb*927.3.2930) mRNA is highly enriched in the DRBD13 bound fraction (~18-fold enrichment relative to the control, whole cell lysate) (Fig. 1B). Interestingly, AREs can be found in the 3′UTR of *RBP6* mRNA itself which is in agreement with the interaction of DRBD13 with AREs in the 3′UTR of targeted mRNAs. Together, these data indicate that DRBD13 binds and potentially regulate transcripts encoding surface coat proteins and *RBP6* mRNA.

3.2. DRBD13 is a cytoplasmic protein and changes in its expression level has severe effects on the cell morphology

To localize DRBD13 in PC trypanosomes, we generated two cell lines expressing N-terminal or C-terminal myc-tagged DRBD13. In both cell lines, DRBD13 was ectopically expressed and localized after 24 h of induction (Fig. 2A). Myc-tagged DRBD13 was absent from the nucleus and showed a homogenous distribution in the cytoplasm. Consistent with these results, Western analyses showed the expression of tagged protein after 24 and 48 h of tet induction, which was absent in wild type (WT) or uninduced (-tet) control cell lines (Fig. 2B).

To examine the effect of DRBD13 expression on PC trypanosomes over time, we ectopically expressed the C-terminal myc-tagged DRBD13. We could detect the expression of DRBD13 on a Western blot only after 3 h of induction by tet addition (Fig. 3B). Two days after induction of DRBD13 expression, we observed slower growth rate as compared to the -tet or WT cells (Fig. 3A). Similar slow growth was observed with N-terminal myc-tagged protein (not shown). Additionally, majority of cells showed changes in morphology after 24 h of induction of both C

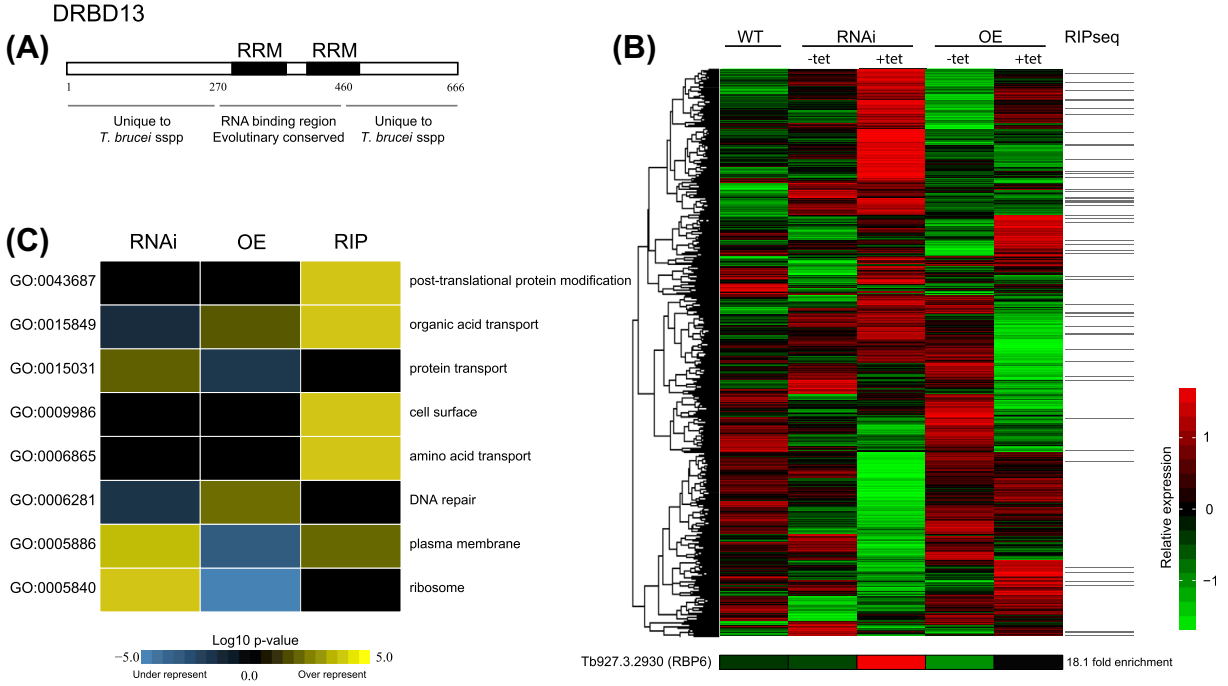

Fig. 1. Sequence and functional characteristics of DRBD13 RBP. (A) DRBD13 protein has a conserved RNA binding region surrounded by two flanking regions that are unique to *T. brucei* sspp. (B) Clustered RNAseq data of 29–13 wild type cells (WT), *DRBD13* knockdown (*RNAi*) or over-expression (OE) presented in a heat map with RIPseq bound transcripts aligned (transcripts with greater than fivefold enrichment in the bound fraction compared to control experiment were considered as bound transcripts). More specifically, details of changes in *RBP6* transcript expression in response to DRBD13 over-expression and knock-down along with its fold enrichment in the DRBD13 RIPseq experiment is shown at the bottom of heat map. (C) Gene ontology (GO) enrichment analysis of knockdown, over-expression, and RIPseq results using GOstat web server. The GO analysis was performed by applying Wilcoxon–Mann–Whitney ranked sum test on the ranked lists of transcripts sorted according to their fold changes/enrichments. Figure is pseudo-colored, with only significant GO terms shown. For the transcriptome data, GO terms were considered as significant if: (1) they were enriched in both of the transcriptome datasets (*P*-value <0.05); (2) their pattern (i.e., over-/under-representation) were opposite in the *RNAi* and over-expression experiments. For the RIP-seq data, the considered cut-off threshold was the false discovery rate <0.05.

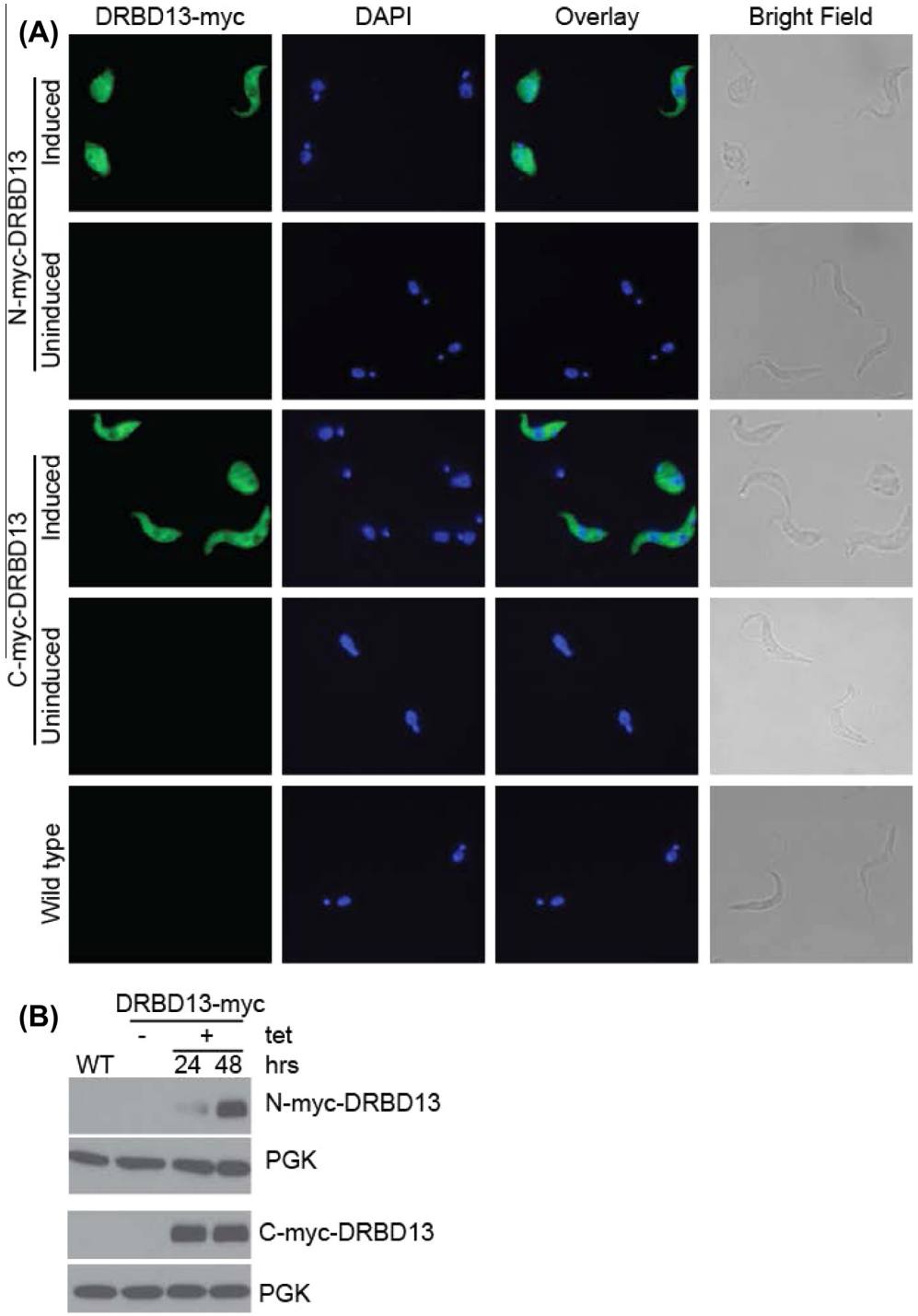

Fig. 2. Localization of DRBD13-myc. (A) N terminal and C terminal myc-tagged DRBD13 expressing cells were used for immunofluorescence experiments. Anti-myc antibody was used to detect DRBD13-myc. Rows 1 and 3 (from top) show a cytoplasmic localization of the tagged protein after 24 h of induction by tetracycline (tet). Rows 2 and 4 (from top) show signals from the -tet cells. The bottom row shows signal from procyclic wild type trypanosomes (WT PC; without the transfected plasmid). DRBD13-myc stands for myc-tagged DRBD13; DAPI was used to stain nuclei and kinetoplasts. (B) Western blot showing the induction of the myc-tagged DRBD13 after 24 and 48 h of induction by tet ('–' uninduced; '+' induced). WT– wild type cells; PGK – phosphoglycerate kinase (loading control).

and N terminal myc-tagged DRBD13 (Fig. 2). The cells however, did not show an apparent cytokinesis defects, as evident from the DAPI-stained nuclei and kinetoplast (Fig. 2A). The effect of DRBD13 over-expression on the cell phenotype and growth was reversible, *i.e.* once DRBD13-myc over-expression was diminished, the trypanosome morphology and growth became normal. Moreover, the growth recovery of the over-expressing cells after day 3 was directly correlated with the decrease in DRBD13 ectopic expression from day 3 onwards (Fig. 3B). These data suggest that the normal level of DRBD13 is in fact crucial for the cells.

A more dramatic effect of knockdown on cell growth was observed at a later time point as compared to the effect seen after over-expression of DRBD13. Knockdown of *DRBD13* following *RNAi* induction after 3 days was lethal to the PC cells (Fig. 3A). The effect

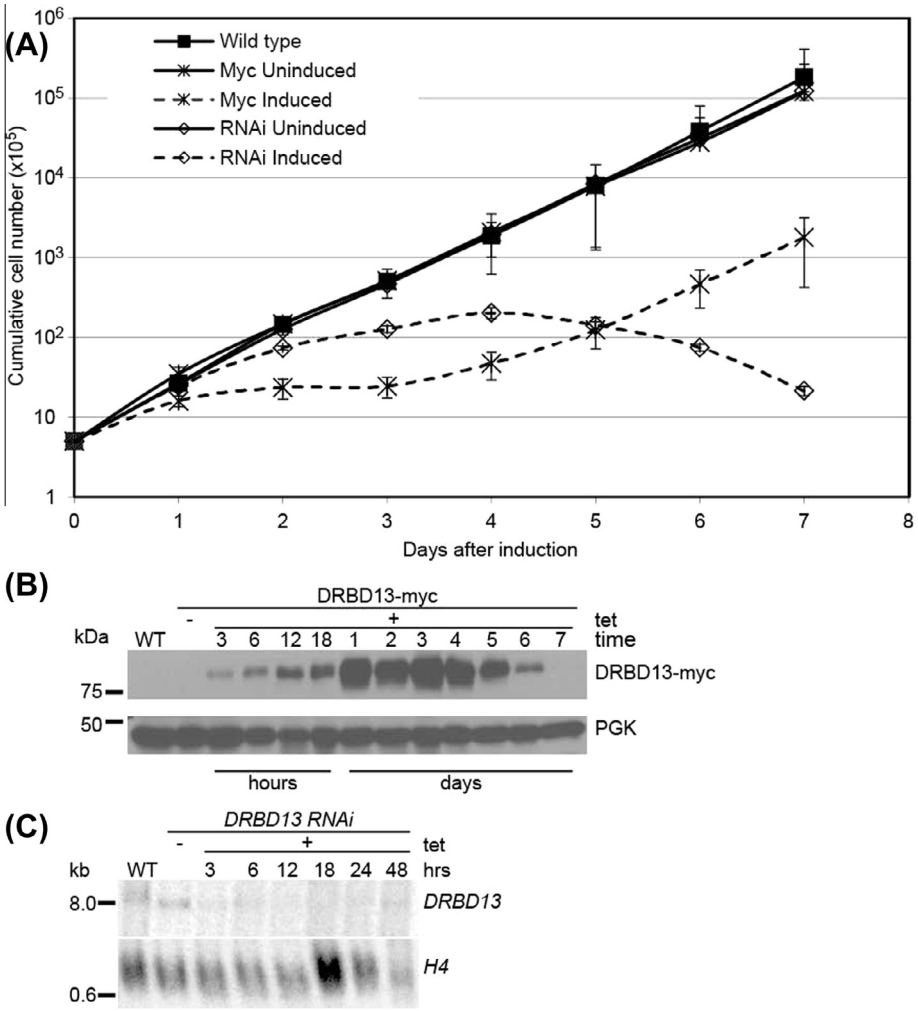

Fig. 3. DRBD13 is an essential protein for normal growth of the procyclic trypanosomes. (A) Growth curve of PC trypanosomes after DRBD13 over-expression and *RNAi*. Number of days after induction by tet addition have been plotted on *x*-axis while *y*-axis shows cumulative cell number. Growth defects arising after *RNAi* and over-expression can easily be seen. It should be noted that the cells recover from growth defect arising after DRBD13 over-expression, while *DRBD13 RNAi* is lethal in PC trypanosomes. (B) Western blot showing the expression of DRBD13-myc (C terminal) after tet induction. The ectopic expression of the tagged protein can be seen after 3 h of induction. Figure depicts time in 'hours (h)' for the first few lanes and in 'days' for the next lanes. By day 7, expression of the DRBD13-myc is completely diminished. Comparison with Fig. A shows that rescue from growth defect in over-expression cell line follows similar pattern as the down-regulation of DRBD13-myc. PGK serves as a loading control. WT – wild type; '–' uninduced; '+' induced. (C) Northern blot showing *DRBD13 RNAi*. Although RNAi does not seem to be completely efficient, it is enough to cause a lethal effect in PC trypanosomes. *Histone H4* (*H4*) mRNA was probed as a loading control. WT- wild type; '–' uninduced; '+' induced.

of knockdown was observed at the *DRBD13* transcript level after 3 h (Fig. 3C). Induction of *DRBD13 RNAi* also affected trypanosome morphology (not shown).

These results suggest the existence of tight regulation on *DRBD13* expression levels in trypanosomes. They also support the putative role of DRBD13 RBP in the regulation of membrane associated proteins.

3.3. Down-regulation or up-regulation effect of DRBD13 on RBP6 mRNA

To examine the effects of DRBD13 expression changes on its target mRNAs and experimentally validate the predicted targets, we used *DRBD13 RNAi* and over-expression cell lines and monitored *RBP6* mRNA levels by Northern blotting (Fig. 4). There was a noticeable effect of *DRBD13 RNAi* on *RBP6* mRNA expression. After 6 h of induction of *DRBD13 RNAi*, the decrease in *DRBD13* mRNA parallels an increase in *RBP6* mRNA level, which further increased after 24 h by 10-fold compared to the WT or uninduced cell lines (Fig. 4A). Increase in the target transcript level after *DRBD13 RNAi*, suggest that DRBD13 destabilizes *RBP6* mRNA. Consistently, although *RBP6* steady state mRNA level is very low in the PC cells, we detected a further reduction (~1–2-fold down-regulation) of *RBP6* mRNA upon DRBD13 over-expression (Fig. 4B). A reduction in *RBP6* mRNA level in -tet with respect to the WT may reflect a leaky expression of DRBD13-myc, which we did not detect on the Western blot. Additionally, as already stated, a low level of *RBP6* mRNA might also have been responsible for the poor quantitation of the radioactive signals. Together, these data suggest that DRBD13 negatively regulates *RBP6* mRNA.

3.4. Tethering assay suggests that DRBD13 destabilizes its bound targets

We employed an RNA tethering approach to assess the possible destabilizing role of DRBD13 on its targets. The experiment was performed as described before [24]. Fig. 5A is a schematic representation of the tethering experiment using two PC cell lines that we generated. One cell line constitutively expressed a *CAT* mRNA containing the "boxB" binding site for bacteriophage lambda N

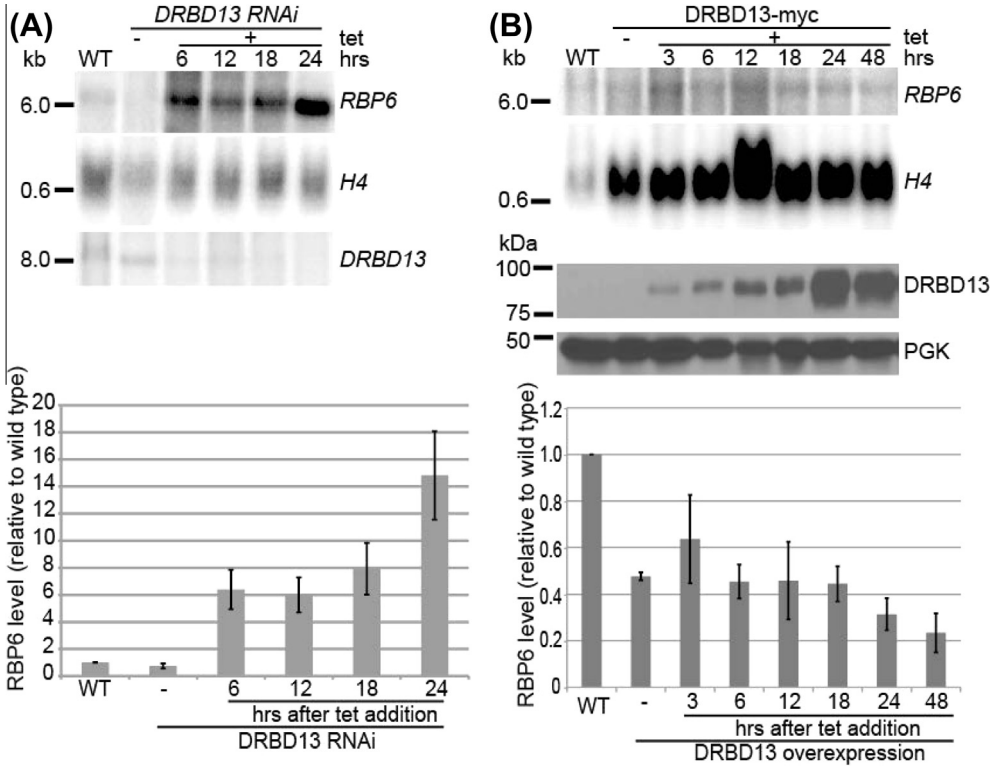

Fig. 4. Effects of changes in DRBD13 level on *RBP6* mRNA. (A) Northern blot showing changes in *RBP6* mRNA level after *DRBD13* knockdown. Top panel shows that the *RBP6* mRNA is significantly up-regulated after 24 h of *DRBD13 RNAi*. *Histone H4* mRNA (*H4*) was used as a loading control to measure the relative amounts of *RBP6* mRNA. Northern blot also shows a successful *DRDB13 RNAi*. Bottom panel of the figure shows a quantitation of the blot. (B) Top panel shows a Northern blot for the *RBP6* mRNA level changes after *DRBD13*-myc expression. Similar to Fig. A, *Histone H4* mRNA (*H4*) was used as a loading control to measure the relative amounts of *RBP6* mRNA. The middle panel is a Western blot showing the expression of *DRBD13*-myc. The blot was probed with anti-myc antibody and anti-PGK antibody (loading control). Bottom panel of the figure shows a quantitation of the *RBP6* mRNA levels similar to Fig. A.

protein (λN) for tethering an inducible λN-DRBD13-myc fusion protein. A second cell line, which is used as control, constitutively expressed the same *CAT* mRNA lacking the "boxB" but expressed the inducible λN-DRBD13-myc protein. The 3′ UTR used in the CAT constructs was from the actin mRNA, which is unresponsive to alterations in the DRBD13 expression. We also verified the cytoplasmic localization of the λN-DRBD13-myc (both tethered and non-tethered) using anti-myc antibody (Fig. S1A). We next examined whether λN-DRBD13-myc fusion protein in these two cell lines alters the *CAT* expression at both the mRNA and the protein levels. When the cell line that constitutively expressed a *CAT* mRNA containing the "boxB" and the λN-DRBD13-myc fusion protein was induced for 6, 12, 18, and 24 h, the *CAT* expression at both the mRNA and the protein levels were significantly reduced (Fig. 5B and C; right panel). In contrast, in the control cell line in the absence of boxB, induction of the λN-DRBD13-myc fusion protein did not lead to any changes in the *CAT* expression at either the mRNA or protein levels (Fig. 5B and C; left panel). These data indicate that the boxB-dependent binding of DRBD13 to 3′ UTR of *CAT* mRNA can lead to destabilization of the *CAT* reporter expression.

To assess whether or not DRBD13 is associated with the polysome-bound mRNAs, extracts from DRBD13 over-expressing cells were subjected to sucrose gradient fractionation and protein distribution was monitored by Western blotting (Fig. S1B). Consistent with the result of tethering assay, DRBD13 was detected in the top fractions 1–3 which presumably represent non-complexed soluble protein fractions away from the fractions containing the small ribosomal protein S9, indicating that DRBD13 is not associated with the actively translating mRNAs.

We conclude that DRBD13 may have a destabilizing effect on its target mRNAs. Nevertheless, we are aware that RBPs do not mediate their regulatory function independent of the other regulators and there is a complex combinatorial interaction network between them. Therefore, it is possible by forcing the binding of an RBP to a transcript that is not its natural target, the interacting partners of the RBP and, consequently, the regulatory effect of the RBP changes.

3.5. Changes in DRBD13 expression level resembles the cell surface remodeling that occurs during the differentiation in the fly

So far, our results suggest that expression of RBP6 and potentially cell membrane proteins are regulated by DRBD13. To investigate the effect of DRBD13 on expression of membrane proteins, we measured changes induced by DRBD13 on key cell membrane proteins of PC and BS life stages. As representatives of these life stages, we selected PC-specific GPEET2 procyclin [14] and VSG, the major BS-specific surface coat protein. To monitor changes induced by DRBD13 on the transcripts encoding these proteins, we conducted two separate time-point experiments in *DRBD13* knock-down and over-expression backgrounds. Intriguingly, we found that GPEET2 is upregulated in response to *DRBD13* knock-down, but VSG remained unchanged (Fig. 6A). Consistently, GPEET2 was down-regulated by over-expression of DRBD13, indicating the destabilizing role of DRBD13 on this important PC cell surface glycoprotein (Fig. 6B). More interestingly, VSG transcripts were detected after 24 h over-expression of DRBD13 (Fig. 6B). In MC life stage, VSG expression is mainly regulated by

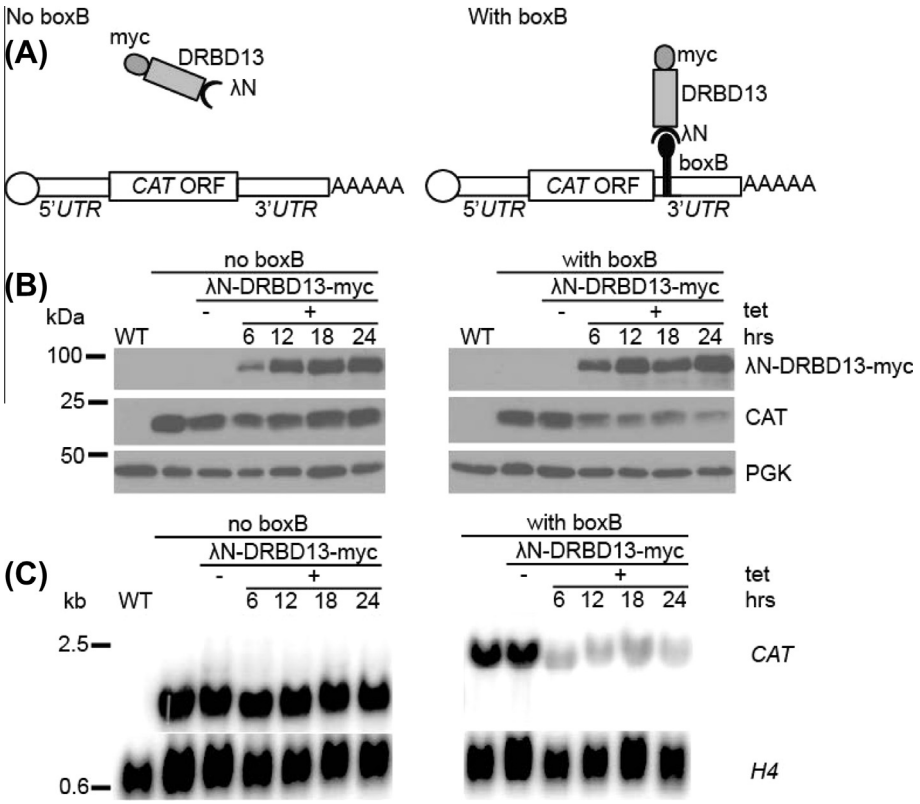

Fig. 5. DRBD13 tethering causes a decrease in reporter mRNA level. (A) Schematic representation of the RNA tethering strategy. The fusion protein (λN-DRBD13-myc) does not bind to the reporter mRNA lacking the recognition sequence for the λN peptide "boxB" in the reporter (left panel). Right panel shows the interaction between the fusion protein 'λN-DRBD13-myc' and the reporter mRNA (mediated by boxB in the 3′UTR). For simplicity only one stem loop structure has been shown in the reporter, although it has 5 tandem boxB sequences. (B) Top panel shows a Western blot to detect the induction of the fusion protein λN-DRBD13-myc. Medium panel shows a down-regulation of CAT expression at protein level in cell lines containing the reporter with boxB sequence (right lanes; 6 h onwards). Left lanes show no significant effect on CAT levels after the induction of the fusion protein in the cell line lacking boxB in the reporter mRNA. Bottom panel shows PGK signal which served as a loading control for the Western blot. WT-wild type; '−' uninduced; '+' induced. (C) Northern blot showing the effect of λN-DRBD13-myc tethering on *CAT* mRNA expression. Consistent with the Western blot, a decrease in *CAT* mRNA can be seen after 6 h onwards (after tet addition) in cell lines with boxB containing reporter (right lanes). As expected, no effect was seen in the boxB deficient reporter's expression (left lanes). Lower panel shows *Histone H4* (*H4*) expression (loading control). WT – wild type; '−' uninduced; '+' induced.

the activity of metacyclic-specific promoters. To determine whether the observed results is limited to the activation of the metacyclic promoter, or the regulation has a more general effect, we assessed the expression patterns of two other BS-specific cell membrane-related proteins, ISG65 [29] and GPI-PLC [15]. Unlike VSG, these two transcripts are expressed in a polycistronic manner by RNA polymerase II. Therefore, putatively, there is no regulation of these two transcripts at the transcriptional level. Consistent with VSG expression pattern, we found that these two transcripts encoding cell membrane-related proteins are also up-regulated in DRBD13-myc over-expression background. Post-translational regulatory pathways play important roles in regulation of *T. brucei* differentiation process [30–33], cell-membrane dynamics (including the regulation of VSG [34] and ISG proteins [35]), and translation [36]. Our RIP-seq experiment suggested preferential association of DRBD13 protein with transcripts involved in post-translational regulation. Therefore, DRBD13 might mediate its effect on the cell by regulation of post-translational regulatory pathways. Overall, these results indicate that expression of DRBD13 is correlated with the expression of BS-specific cell coat proteins, but negatively correlated with those of PC life stage.

4. Conclusions

Recent progress in post-transcriptional gene regulation in *T. brucei* and its critical role in development highlights the importance of RBPs and their interactions with *cis*-regulatory elements. A remarkable example of such regulation is when over-expression of an ectopic RBP6 in PC trypanosomes led to appearance of mammalian-infective MC forms expressing the VSG coat [22]. However, the exact mechanism of these events is unclear. Because DRBD13 associates with ARE containing transcripts encoding surface coat proteins and *RBP6* transcript is among the significantly enriched targets in DRBD13 RIP-seq data, we sought to determine how changes in DRBD13 expression may influence cell morphology and *RBP6* mRNA. Significantly, our experiments revealed that DRBD13 acts as a negative regulator for RBP6 and also major PC cell surface proteins like GPEET2. However, more interestingly, the expression of DRBD13 is positively correlated with the appearance of BS-specific cell surface genes such as VSG and ISG. To assess the biological relevance of DRBD13 over-expression, we re-analyzed the *T. brucei* transcriptome in infected tse-tse tissues [22]. Consistently, we found that DRBD13 protein is up-regulated (∼2-fold up-regulation) in the proventriculus life stage of the parasite. More interestingly, the expression of *RBP6* and procyclin transcripts are down-regulated in the MC compared to the proventriculus life stage which can be, at least partially, a delayed response to the over-expression of *DRBD13* transcript in the proventriculus life stage. Additionally, the observed impairment between RBP6 and VSG expressions in our experiments suggests that RBP6 is not the direct regulator of VSG transcripts, and some intermediate factors are involved in between. Overall, we speculate that DRBD13 is one of the major regulators that coordinate the expression changes of at least cell

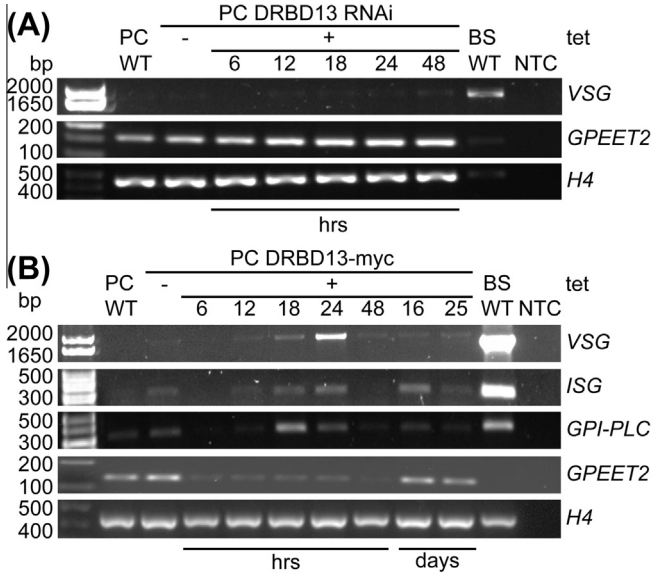

Fig. 6. DRBD13 mediates the cell surface remodeling of PC cells. (A) RNA was isolated at various time points after *DRBD13*-RNAi induction. cDNA was prepared using random primers followed by semi-quantitative reverse transcription PCR. Over time, we observe a clear increase in the *GPEET2 procyclin* levels. However, no change in expression of BS-specific VSG was observed. *Histone H4* (*H4*) primers were used for PCR to show equal amount and the quality of input cDNA. NTC- no template control, WT – wild type; '–' uninduced; '+' induced. (B) RNA was isolated at various time points after DRBD13-myc induction. cDNA was prepared using random primers followed by semi-quantitative reverse transcription PCR. A prominent band showing *VSG*, *ISG*, and *GPI-PLC* expression in PC trypanosomes was detectable after 18–24 h of DRBD13-myc induction. A clear decrease in the *GPEET2 procyclin* levels can also be seen after 6 h of DRBD13-myc expression. At later time points (16 and 25 days) *GPEET2 procyclin* expression becomes normal. NTC – no template control, WT – wild type; '–' uninduced; '+' induced. *Histone H4* (*H4*) primers were used for PCR to show equal amount and the quality of input cDNA.

membrane transcripts and *RBP6* mRNA during the developmental progression in the insect-stage.

Conflict of interest

None.

Acknowledgements

We thank Dr. Christine Clayton for the vectors (myc tagging and tethering), 1313 PC cell lines and anti-S9 antibodies. We also thank Dr. Marilyn Parsons for anti-PGK antibody. This work was supported by Canadian Institute of Health Research (CIHR) grant 119464 (to R.S.) and Le Fonds Quebecois de la recherche sur la nature et les technologies (FQRNT; for Centre for Host-Parasite Interactions and research at Institute of Parasitology). V.H.G. is supported by a CIHR Systems Biology Fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.05.036.

References

[1] Steverding, D. (2008) The history of African trypanosomiasis. Parasites Vectors 1, 3.
[2] Matthews, K.R. (2005) The developmental cell biology of *Trypanosoma brucei*. J. Cell Sci. 118, 283–290.
[3] Vickerman, K., Tetley, L., Hendry, K.A. and Turner, C.M. (1988) Biology of African trypanosomes in the tsetse fly. Biol. Cell 64, 109–119.
[4] Sharma, R., Gluenz, E., Peacock, L., Gibson, W., Gull, K. and Carrington, M. (2009) The heart of darkness: growth and form of *Trypanosoma brucei* in the tsetse fly. Trends Parasitol. 25, 517–524.
[5] Matthews, K.R., Ellis, J.R. and Paterou, A. (2004) Molecular regulation of the life cycle of African trypanosomes. Trends Parasitol. 20, 40–47.
[6] Ziegelbauer, K. and Overath, P. (1992) Identification of invariant surface glycoproteins in the bloodstream stage of *Trypanosoma brucei*. J. Biol. Chem. 267, 10791–10796.
[7] Ziegelbauer, K., Multhaup, G. and Overath, P. (1992) Molecular characterization of two invariant surface glycoproteins specific for the bloodstream stage of *Trypanosoma brucei*. J. Biol. Chem. 267, 10797–10803.
[8] Roditi, I., Schwarz, H., Pearson, T.W., Beecroft, R.P., Liu, M.K., Richardson, J.P., Buhring, H.J., Pleiss, J., Bulow, R., Williams, R.O., et al. (1989) Procyclin gene expression and loss of the variant surface glycoprotein during differentiation of *Trypanosoma brucei*. J. Cell Biol. 108, 737–746.
[9] Clayton, C.E. (2002) Life without transcriptional control? From fly to man and back again. EMBO J. 21, 1881–1888.
[10] Fernandez-Moya, S.M. and Estevez, A.M. (2010) Posttranscriptional control and the role of RNA-binding proteins in gene regulation in trypanosomatid protozoan parasites. Wiley Interdiscip. Rev. RNA 1, 34–46.
[11] Clayton, C. (2012) MRNA turnover in trypanosomes. Nucleic Acids Mol. Biol. 28, 79–97.
[12] Peng, S.S., Chen, C.Y., Xu, N. and Shyu, A.B. (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. EMBO J. 17, 3461–3470.
[13] Fan, X.C. and Steitz, J.A. (1998) Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. EMBO J. 17, 3448–3460.
[14] Acosta-Serrano, A., Vassella, E., Liniger, M., Kunz Renggli, C., Brun, R., Roditi, I. and Englund, P.T. (2001) The surface coat of procyclic *Trypanosoma brucei*: programmed expression and proteolytic cleavage of procyclin in the tsetse fly. Proc. Natl. Acad. Sci. U.S.A. 98, 1513–1518.
[15] Gruszynski, A.E., van Deursen, F.J., Albareda, M.C., Best, A., Chaudhary, K., Cliffe, L.J., del Rio, L., Dunn, J.D., Ellis, L., Evans, K.J., Figueiredo, J.M., Malmquist, N.A., Omosun, Y., Palenchar, J.B., Prickett, S., Punkosdy, G.A., van Dooren, G., Wang, Q., Menon, A.K., Matthews, K.R. and Bangs, J.D. (2006) Regulation of surface coat exchange by differentiating African trypanosomes. Mol. Biochem. Parasitol. 147, 211–223.
[16] Stoecklin, G., Stoeckle, P., Lu, M., Muehlemann, O. and Moroni, C. (2001) Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements. RNA 7, 1578–1588.
[17] DeMaria, C.T. and Brewer, G. (1996) AUF1 binding affinity to A + U-rich elements correlates with rapid mRNA degradation. J. Biol. Chem. 271, 12179–12184.
[18] Piecyk, M., Wax, S., Beck, A.R., Kedersha, N., Gupta, M., Maritim, B., Chen, S., Gueydan, C., Kruys, V., Streuli, M. and Anderson, P. (2000) TIA-1 is a translational silencer that selectively regulates the expression of TNF-alpha. EMBO J. 19, 4154–4163.
[19] Lai, W.S., Carballo, E., Strum, J.R., Kennington, E.A., Phillips, R.S. and Blackshear, P.J. (1999) Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Mol. Cell. Biol. 19, 4311–4323.
[20] Najafabadi, H.S., Lu, Z., MacPherson, C., Mehta, V., Adoue, V., Pastinen, T. and Salavati, R. (2013) Global identification of conserved post-transcriptional regulatory programs in trypanosomatids. Nucleic Acids Res. 41, 8591–8600.
[21] Wurst, M., Robles, A., Po, J., Luu, V.D., Brems, S., Marentije, M., Stoitsova, S., Quijada, L., Hoheisel, J., Stewart, M., Hartmann, C. and Clayton, C. (2009) An RNAi screen of the RRM-domain proteins of *Trypanosoma brucei*. Mol. Biochem. Parasitol. 163, 61–65.
[22] Kolev, N.G., Ramey-Butler, K., Cross, G.A., Ullu, E. and Tschudi, C. (2012) Developmental progression to infectivity in *Trypanosoma brucei* triggered by an RNA-binding protein. Science 338, 1352–1353.
[23] Estevez, A.M., Kempf, T. and Clayton, C. (2001) The exosome of *Trypanosoma brucei*. EMBO J. 20, 3831–3839.
[24] Delhi, P., Queiroz, R., Inchaustegui, D., Carrington, M. and Clayton, C. (2011) Is there a classical nonsense-mediated decay pathway in trypanosomes? PLoS ONE 6, e25112.
[25] Cristodero, M. and Clayton, C.E. (2007) Trypanosome MTR4 is involved in rRNA processing. Nucleic Acids Res. 35, 7023–7030.
[26] Haile, S., Cristodero, M., Clayton, C. and Estevez, A.M. (2007) The subcellular localisation of trypanosome RRP6 and its association with the exosome. Mol. Biochem. Parasitol. 151, 52–58.
[27] Djikeng, A., Shi, H., Tschudi, C., Shen, S. and Ullu, E. (2003) An siRNA ribonucleoprotein is found associated with polyribosomes in *Trypanosoma brucei*. RNA 9, 802–808.
[28] Jensen, B.C., Sivam, D., Kifer, C.T., Myler, P.J. and Parsons, M. (2009) Widespread variation in transcript abundance within and across developmental stages of *Trypanosoma brucei*. BMC Genomics 10, 482.
[29] Overath, P., Chaudhri, M., Steverding, D. and Ziegelbauer, K. (1994) Invariant surface proteins in bloodstream forms of *Trypanosoma brucei*. Parasitol. Today 10, 53–58.
[30] Vassella, E., Kramer, R., Turner, C.M., Wankell, M., Modes, C., van den Bogaard, M. and Boshart, M. (2001) Deletion of a novel protein kinase with PX and FYVE-related domains increases the rate of differentiation of *Trypanosoma brucei*. Mol. Microbiol. 41, 33–46.

[31] Domenicali Pfister, D., Burkard, G., Morand, S., Renggli, C.K., Roditi, I. and Vassella, E. (2006) A Mitogen-activated protein kinase controls differentiation of bloodstream forms of *Trypanosoma brucei*. Eukaryot. Cell 5, 1126–1135.

[32] Barquilla, A., Saldivia, M., Diaz, R., Bart, J.M., Vidal, I., Calvo, E., Hall, M.N. and Navarro, M. (2012) Third target of rapamycin complex negatively regulates development of quiescence in *Trypanosoma brucei*. Proc. Natl. Acad. Sci. U.S.A. 109, 14399–14404.

[33] Urbaniak, M.D., Martin, D.M. and Ferguson, M.A. (2013) Global quantitative SILAC phosphoproteomics reveals differential phosphorylation is widespread between the procyclic and bloodstream form lifecycle stages of *Trypanosoma brucei*. J. Proteome Res. 12, 2233–2244.

[34] Lopez-Farfan, D., Bart, J.M., Rojas-Barros, D.I. and Navarro, M. (2014) SUMOylation by the E3 ligase TbSIZ1/PIAS1 positively regulates VSG expression in *Trypanosoma brucei*. PLoS Pathog. 10, e1004545.

[35] Leung, K.F., Riley, F.S., Carrington, M. and Field, M.C. (2011) Ubiquitylation and developmental regulation of invariant surface protein expression in trypanosomes. Eukaryot. Cell 7, 916–931.

[36] Pereira, M.M., Malvezzi, A.M., Nascimento, L.M., Lima, T.D., Alves, V.S., Palma, M.L., Freire, E.R., Moura, D.M., Reis, C.R. and de Melo Neto, O.P. (2013) The eIF4E subunits of two distinct trypanosomatid eIF4F complexes are subjected to differential post-translational modifications associated to distinct growth phases in culture. Mol. Biochem. Parasitol. 190, 82–86.